Iranian Journal of Veterinary Science and Technology

Received: 2018- Jun-11
Accepted after revision: 2018- Oct-13
Published online: 2019-

RESEARCH ARTICLE

DOI: 10.22067/veterinary.v2i10.73451

Evaluation the latex agglutination test for detection of the causative agents of bacterial mastitis in milk samples

Mohammad Khosravi[a], Mohammad Bafandeh Dehaghi[b], Saad Gooraninezhad[c], Darioush Gharibi[a], Masood Ghorbanpoor[a]

[a] Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz , Iran. [b] Graduate Student of Veterinary Medicine, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Iran. [c] Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Iran.

ABSTRACT

Mastitis is the main disease in dairy farms which causes serious losses. The early diagnosis and specific treatment can prevent the spread of the disease and the economic losses. The aim of this study was to evaluate the accuracy of the latex agglutination assay for detection of the main bacterial mastitis agent. The antiserum against Staphylococcus aureus, Trueperella pyogenes, Streptococcus agalactiae and Escherichia coli were prepared from immunized rabbits. The couplings of antibodies to latex particles were optimized and after that, the limit of detection (LOD) of latex agglutination test was evaluated for detection of the mentioned bacteria. The detection limit for the Streptococcus agalactiae, E. coli, Staphylococcus aureus and T. pyogenes were respectively 1.3×103, 2×107, 1.58×104 and 5.4×104 colony-forming unit per each milliliter of the bacterial suspensions. The sensitivity of the latex test has a noticeable reduction in bacterial detection in milk compression to normal saline.This fast method can be used for detection of the mentioned bacteria in the bacterial cultures and milk samples; the latex agglutination test could be evaluated as a fast, cost benefit and practical method in dairy farms.

Keywords

Mastitis; Agglutination; Latex

Abbreviations

??????

Corresponding author:
Mohammad Khosravi
Tell/Fax:
Email: m.khosravi@scu.ac.ir

Introduction

Mastitis is inflammation of the mammary glands; it is associated with physical and chemical changes of the milk and pathological changes in udder glandular tissue. The quality and quantity of milk production are dependent to the mammary tissues' states, the secretary cells' efficiency and nutritious availability. Mastitis is associated with decreases of the milk secretion and changes of the milk component [1]. Mastitis is one of the world wide most important diseases in dairy cattle herds; as is fitted to the most important factor in the breeding failure. Mastitis could reduce the cow price, herd's income and profits. The economic impact of the mastitis divided to the direct effects (medicinal treatment, veterinary expenses and mortality) and indirect effects (decrease the milk production or reduce the protein concentration in the produced milk, increase the reproductive failure rate and reduce the survival rate) [2, 3].

Mastitis caused by various factors such as infectious agents, mechanical or chemical trauma. The infectious agents of mastitis include bacterial agents, viruses and fungi [2]. The main cause of the bacterial mastitis is invasion and toxin production of the bacteria in mammary glands; for this reason, the mastitis treatments are based on the antibiotics therapy [4]. The main bacterial mastitis are generally caused by; Streptococcus agalactiae, Streptococcus dysgalactiae, Staphylococcus aureus, Trueperella pyogenes, Mycoplasma bovis, Streptococcus uberis and Coliform bacteria [5].

Detection of the causative agents of bacterial mastitis is necessary for accurate treatment, management, and appropriate antibiotics therapy. The microbial culture is conventional and general route for bacterial detection; this method is time-consuming; initial diagnosis needs at least 24 to 48 hours [6]. However, rapid detection and immediately handling are critical issues in management of the clinical mastitis; delay would results in increase of the therapy period and extensive antibiotics utilization [7]. In addition, interpretation of the cultivated plates is associated with challenges; there are some differences on diagnostic results of the laboratories, although they use same and standardized methods [8].

Enzyme-linked immune-sorbent assay (ELISA) is another method for detection of the bacterial agents of mastitis; but despite the abundant advantage, it failed to detect the low concentration of some antigens [9]. Another routine method that used for diagnosis of the causative agents of mastitis is Polymerase chain reaction (PCR); the main weakness of the conventional PCR is utilization of the agarose gel with very weak clarity and precision [10]. The other difficulty in mastitis diagnosis by using PCR and ELISA are the limited use at farm, the high cost and requirement of the expert. The number of somatic cells does not always communicate with mastitis; rhe elevation of the somatic cell count (SCC) may be occurred due to the problems other than mastitis such as lactation, the level of the milk production, the number of the milking, stress, seasons, and the animals breed [11]. Other disadvantages of the SCC method are the required high time, expensive equipment and cost [12]. The electrical conductivity of milk could be a useful diagnosing method for detection of the mastitis, but due to the large number of false positive, could not be a reliable assay [13]. The milk production industry and animal husbandry farms are searching for the alternative fast and accurate diagnostic methods for mastitis. At now, the farms are bigger than past and they produce more milk; these conditions needs automatic milking machines, processor and hygienic environment. Exclusion of the disadvantage methods is an important section of the farm management [14].

Latex particles have been employed on the immunoassay and related biomedical diagnostic methods. Each of the attached antigens or antibodies to the latex particles on proper ratio gives visible sediment in a few minutes. Latex particles uptake antigen/antibody and suspend it on the surface; so when acquainted with their specific antigen/antibody, the reaction is easily visible as physical accumulation [15].

The latex agglutination assays have suitable sensitivity and specificity, affordable, required little skills, tools and time. It could be a good alternative method for detection of the bacterial mastitis agents. This research evaluated the sensitivity and accuracy of the latex agglutination test for detection of the selected bacterial mastitis agents.

Results

The rabbits produced a proper antibody titer after three sets of immunization; however S. agalactiae and S. aureus have a greater antibacterial antibody titer on micro-agglutination test (Table 1) than other. The SDS-PAGE analysis showed appropriate purification of the antibody by using ion-exchange chromatography (Figure 1).

The antibacterial antibody titers of the purified IgG were analyzed using micro-agglutination test. The results (Table 1) showed the failed whole extraction of the monovalent antibody using affinity purification.

Coupling of antibodies to latex

The bacterial latex at optimum conditions of the antibody-latex coupling showed appropriate visible positive and negative agglutination after 30-180 seconds. The stability test showed excellent results at least for six months (Figure 2).

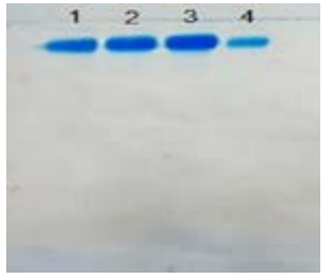

Figure 1
Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of the purified IgG by ion exchange chromatography. The four clearly bands depicted good efficacy of the methods. 1. T. pyogenes, 2. S. agalactiae, 3. S. aureus and 4. E. coli

Table 1
The antibacterial antibody titers of the purified IgG by Ion Exchange chromatography and affinity purification.

Bacteria	E. coli	S. aureus	S. agalactiae	T. pyogenes
Ion exchange	512	1024	1024	512
Affinity purification	256	256	128	512

The sensitivity latex agglutination test for diagnosis of bacterial mastitis

Ten number of the 1/10 serial dilutions of the bacteria were prepared from initial concentration adjusted to 2 MacFarland standard in normal saline and sterilized bovine milk; the 2 final dilutions were cultured on blood agar for accurate assessment of the bacterial count. The agglutinated reactions were graded +1 to +4 based on the time and severity of the reactions. As seen in table 2, different sensitivity observed in the 4 bacterial latex reactions; the sensitivity directly correlated to the antibacterial antibody titer of the corresponding hyper-immune sera (Table 1).

The detection limit of the bacterial number in milk and normal saline samples were calculated according to the culture method. The detection limit of the bacterial latex has been noticeably reduced in milk samples than normal saline (Table 2). Incubation of the polluted milk samples at 37°C for 30-120 minutes, enhance the detection limit 10-100 times; however, incubation at 37°C more than 120 minutes lead to coagulation of the milk samples. Also, the essential bacterial number for induction of the positive reaction decreased 10 times by using specific antibody instead of the total IgG.

The total IgG against different bacteria mixed at the same concentration and attached to the latex particles. The produced multi-bacterial latex could agglutinate four types of the corresponding bacteria at different detection limits. These results (Table 2) have direct correlation with the corresponding antibacterial antibody titer of the hyper-immune sera.

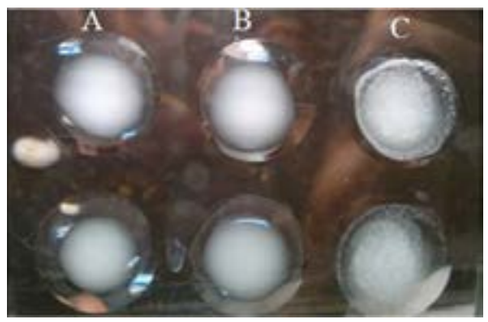

Figure 2
The stability of the prepared bacterial latex after six months. A and B) negative results of the control samples and C) positive results of the samples.

Discussion

In current study the main bacterial agents of mastitis were detected by latex agglutination test, successfully. The detection limits of these latex agglutination tests were dependent to the antibacterial antibodies titer in the corresponding serum. The detection limit was 1.3×103, 2×107, 1.58×104 and 5.4×104 colony forming unit per each milliliter (CFU/ml) of the normal saline for Streptococcus agalactiae, E. coli, Staphylococcus aureus and T. pyogenes respectively.

The studies that utilized sensitized latex for detection of bacteria were noted in the flowing. Hechemy et al. [17] coupled the anti E. coli IgG to the latex particles; the obtained sensitivity was 1.5-5.7×106 CFU/ml. As the same, Hajra et al [18] reported detection of E. coli at 5×106-5×107 CFU/ml by latex agglutination. The sensitivity of the latex test for detection of the Streptococcus reported as 103 CFU/ml [19].

Sumithra et al [20] reported a detection limit of 5×104 for Bacillus cereus by use of polyclonal antibody. Based on the results of this study, detection limit of the latex test for bacteria tracking is directly dependent to the level of the specific antibody, affinity and avidity of the used antibody. In comparison with other research, the detection limit of the produced latex for detection of the bacteria in the polluted milks can be reported as appropriate.

Also, current study tried to develop a rapid and easy method for purification of the specific antibacterial polyclonal antibody. However, the antibacterial antibody titer of the purified antibody was 50-60% lower than the total IgG; the affinity purified antibody enhanced the limit detection of the latex tests up to 10 times. Due to the poor efficiency of the current affinity method, the total IgG which purified by Ion Exchange chromatography were preferred in this study.

Table 2
The detection limit of the latex test and multi- latex for detection of bacteria in milk and normal saline samples.

Bacteria	T. pyogenes	S. aureus	E. coli	S. agalactiae
Normal saline	5.4×10^4	1.54×10^4	$107 \times 2 \times 10^7$	1.3×10^3
Milk	4.8×10^7	7.5×10^5	$106 \times 3.9 \times 10^6$	11.28×10^6
Multi-latex in normal saline	6.3×10^4	8×10^4	4×10^7	5.2×10^3

Utilization of a cost benefit and appropriate method for purification of the specific antibodies suggested for commercialization of latex agglutination test for detection of the bacterial infections. The results of other study are also fitted with this proposal. The latex agglutination test for detection of the S. aureus had cross reaction with Staphylococcus saprophyticus [21]. Also, the Staphylococcus aureus and its enterotoxins detection of in milk samples had failed results [22]. Moser et al [23] showed that latex agglutination test has detected the S. aureus with different genomic profile. The previous research often analyzed latex on pure bacteria obtained from culture but the current study reported the sensitivity of the latex test for detection of the main causes of bacterial mastitis in the milk samples, successfully.

In the current study, the best detection limit obtained by employment of the specific antibody, ion exchange purified IgG and ammonium sulfate precipitated antibody, respectively. The polyclonal IgG often used for sensitization of the latex particles; however employment of the monoclonal antibody resulted in a higher specificity [24]. Due to the simple steps of purification, higher level of the affinity and specificity, the IgG isotype often used for sensitization of the latex particles; despite the higher ability of IgM in induction of the agglutination reactions [25].

Conclusion

In conclusion, the results showed appropriate detection limit of the latex agglutination test for detection of the mentioned mastitis agents. Also, in agreement with the previous study, the latex agglutination test in association with the bacterial culture and biochemical tests could help the correct and rapid detection of the bacterial mastitis agents. Finally the current study suggested the use of the high affinity IgG and coloured latex which can enhance the detection limit of the latex agglutination test in milk samples.

Materials and methods

Bacterial preparation

The native isolation of Streptococcus agalactiae (clinical isolate of bovine mastitis), Trueperella pyogenes (clinical isolate of bovine mastitis), Staphylococcus aureus (PTCC: 1431) and Escherichia coli (PTCC: 1399) were prepared from archive section of the microbiology department of the veterinary medicine. The bacteria were cultured in blood agar medium and incubated at 37 ° C for a period of 24-48 hours depending on the type of bacteria. The biochemical tests were used to confirm the identity of the purred bacteria. The obtained bacterial samples were killed by incubation on 95 ° C for 2 hours; in order to ensure the inactivation, the inactivated bacteria were cultured on blood agar medium.

Immunization

The eight male rabbit with average weight of 2±0.2 Kg were divided in 4 groups and maintained according to the animal care division. The 0.5 ml of inactivated bacteria, at concentration adjusted to 4 McFarland standard, mixed with equivalent amount of the complete freund's adjuvant. The prepared antigens were injected subcutaneously and intramuscularly to each rabbit in groups. The booster antigens were prepared by mixing 0.5 ml of the inactivated bacteria at concentration adjusted to 2 McFarland standard with equal amount of the incomplete freund's adjuvant; three sets of booster were injected as before at 2 week intervals (26). The micro-agglutination test was used to assess the antibody antibacterial titer of the each immunized rabbits. The hyper-immune sera were harvested from the immunized rabbits and stored at -20° C. The micro-agglutination test was used to assess the antibacterial titers of the prepared hyper immune sera.

Antibody purification

The IgG antibodies were purified by Ion Exchange chromatography on DEAE-C column (Sigma, Number: D3764) with instructions fitting to the practical immunology book [16]. Purification of the specific antibodies for each bacterium has been done in accordance with the following steps: The 2 mg/ml of purified IgG was mixed with 0.5 ml of the corresponding bacteria at a concentration adjusted to 4 MacFarland standard and incubated at the 37 ° C for one hour, along with shaking. The antigen-antibody complexes were precipitated by centrifugation for 5 minutes with 10000 RPM; the resulting sediments were harvested and the supernatant were used for more purification as the previous steps. The process repeated twice and the precipitated complexes were collected together. The precipitates were mixed with 1 ml phosphate buffered saline (PBS). The suspension pH was adjusted to 3.5 and shacked for 3 minutes. Then the same centrifuges conditions were used for bacterial sedimentation. The supernatant, which contain specific antibody were collected and the pH was adjusted to 7.2. The quantity, purity and titer of the purified antibody were checked by Bradford protein assay, SDS-PAGE and micro-agglutination test, respectively.

Coupling of the antibody to latex particle

Different buffers, pH and temperature conditions were tested for latex activation and antibody coupling to the latex particles. The optimum conditions were optimized as the flowing steps. The latex particle (prepared in our department) 100µl (5 mg) were mixed with 400µl of PBS (pH: 7.2) and 500µl triton 0.02%. The suspension was centrifuged for 8 minutes at 7000 rpm; the precipitated latex were mixed with an equal amount of the PBS and triton and washed twice as before. The washed latex was mixed with a solution containing Cyanogen bromide (200mg/5ml). The pH of the prepared suspension was fixed on 10.5 by adding NaOH 0.3%. The mixture left on electrical shaker for 30 minutes; after that the mixture centrifuged and the activated latex particles washed as before. The precipitated latex mixed with 50 mM MES buffer (pH: 6.1); 750µl IgG (600 µg) were added to the latex suspension. The mixture left on electric shaker for 30 minutes and overnight on 4°C. The bovine serum albumin 250µl (4mg/ml) were added as blocker; the reagents shacked for 1 hours at room temperature. The suspension was centrifuged for 8 minutes at 6000rpm; the precipitated latex mixed with 350 µl of MES buffer containing BSA (4mg/ml). The stability of the produced antibacterial latex tested along to 6 months. In addition, mixtures of the 4 prepared antibodies were attached to latex particles as mentioned above.

Latex Agglutination test

The ready to use bacterial latex (25µl) was poured on agglutination plate and mixed with an equal volume of the various concentrations (Seven concentration of the 1/10 serial dilutions of the bacteria which prepared from initial concentration equal to 1 MacFarland standard in normal saline and sterilized bovine milk) of the tested bacteria. The plate was shacked constantly and the reactions were observed for 4 minutes. Along with the test samples, negative sample of bacteria and latex were used as control. The crossing reactions were tested by combination of the bacterial latex with bacteria other than corresponding bacteria. The sensitivity of the bacterial latex tested for detection of the various numbers of the tested bacteria in polluted milk. Firstly, bacteria were mixed with 1ml PBS at a concentration adjusted to 2 McFarland standard; the bacterial suspensions were centrifuged and the precipitates were mixed with an equal volume of the sterilized milk. Seven sets of 1/10 serial dilution of each bacteria were prepared on sterilized normal milk samples; the 2 last dilution were cultured on blood agar and the bacterial colony were counted (CFU/ml). The ready to use bacterial latex were diluted as 1/3 in MES buffer; the agglutination tests were done by mixing of the diluted latex 75µl with dilutions of the polluted milks (25µl). The negative samples incubated on 37°C and tested each 30 minutes.

Acknowledgment

This study was financially supported by the Shahid Chamran university of Ahvaz grant.

Author Contributions

Conceived and designed the experiments: M.Kh., M.Gh., D.Gh. S.G. Performed the experiments: M.Kh M.B.D., D.Gh Analyzed the data: S.G. Research space and equipment: M.Kh., M.Gh., D.Gh. S.G Contributed reagents/materials/analysis tools: M.Kh., M.Gh., D.Gh. S.G.

Conflict of Interest

Author declares that have no conflict of interest.

References

1. Souto LI, Minagawa CY, Telles EO, Garbuglio MA, Amaku M, Melville PA. Correlation between mastitis occurrence and the count of microorganisms in bulk raw milk of bovine dairy herds in four selective culture media. Journal of Dairy Research. 2010; 77; 63-70.
2. Radositi OM, Gay CC, Blood DC, Hinchcliff KW. Veterinary Medicine 9th edition. Saunders ltd, London 2000; pp: 603-658.
3. Donovan DM, Kerr DE, Wall RJ. Engineering disease resistant cattle. Transgenic Research. 2005; 14; 563-567.
4. Thomsone K, Rantala M, Hautala M, Pyorala S, Kaartinen L. Cross-sectional prospective survey to study indication-based usage of antimicrobials in animals: Results of use in cattle. BMC Veterinary Research. 2008;14; 4-15.
5. Hillerton JE, Berry EA. Treating mastitis in the cow–a tradition or an archaism. Journal of Applied Microbiology. 2005; 98; 1250-1255.
6. Hope A. Laboratory Handbook on Bovine Mastitis. Australian Veterinary Journal. 2000; 78; 488. doi:10.1111/j.1751-0813.2000.tb11869.x
7. Barkema HW, Schukken YH, Zadoks RN. The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine Staphylococcus aureus mastitis. Journal of Dairy Science. 2006; 89; 1877–1895.
8. Pitkala A, Gindonis V, Wallin H, Honkanen-Buzalski T. Inter-laboratory proficiency testing as a tool for improving performance in laboratories diagnosing bovine mastitis. Journal of Dairy Science . 2005; 88; 553–559.
9. Malou N, Raoult D. Immuno-PCR: a promising ultrasensitive diagnostic method to detect antigens and antibodies. Trends in Microbiology. 2011; 19; 295–302.
10. Willey JM. Prescott, Harley, and Klein's Microbiology.7thed. New York: McGraw Hill 2008; 11; 10-88.
11. Schepers AJ. Estimation of variance components for somatic cell counts to determine thresholds for uninfected quarters. Journal of Dairy Science. 1997; 80; 1833–1840.
12. Pilla R. Differential cell count as an alternative method to diagnose dairy cow mastitis. Journal of Dairy Science. 2013; 96; 1653–1660.
13. Hovinen M. Accuracy and reliability of mastitis detection with electrical conductivity and milk colour measurement in automatic milking. Acta Agriculturae Scandinavica Section A-Animal Science. 2006; 56: 121–127.
14. Duarte M, Freitas PP, Bexiga R. Technological advances in bovine mastitis diagnosis: an overview. Journal of veterinary diagnostic investigation. 2015; 27: 665-672.
15. Litwin SD. Agglutination with Antigen on Inert Particles. I. Latex Fixation Technique. Methods in Immunology and Immunochemistry. 1977; 5: 115-120.
16. Hay FC, Westwood OMR. Practical Immunology, fourth ed. Blackwell Publishing Company, Malden. 2002; pp: 14–15.
17. Hechemy K, Stevens RW, Gaafar HA. Detection of Escherichia coli antigens by a latex agglutination test. Applied Microbiology. 1974; 28: 306-311.
18. Hajra TK, Bag PK, Khan A, Das SC, Ramamurthy T, Mukherjee S. Development of a simple latex agglutination assay for detection of shiga toxin-producing Escherichia coli (STEC) by using polyclonalantibody against STEC. Clinical and Vaccine Immunology. 2007; 5: 600-604.
19. Das A, Ray P, Sharma M, Gopalan S. Rapid diagnosis of vaginal carriage of group B beta haemolytic streptococcus by enrichment cum antigen detection test. Indian Journal of Medical Research. 2003; 117: 247-252.
20. Sumithra TG, Chaturvedi VK, Gupta PK, Sunita SC, Rai AK, Kutty MVH, Murugan MS. Development of a simple and rap-

id method for the specific identification of organism causing anthrax by slide latex agglutination. Applied Microbiology. 2014; 58: 401-407.

21. Gregson DB, Low DE, Skulnick M, Simor AE. Problems with rapid agglutination methods for identification of staphylococcus aureus when staphylococcus saprophyticusIs being tested. Journal of Clinical Microbiology. 1988; 26: 1398-1399.

22. EL-Said WA, Gad Morgan SD, El-Shabrawy M, Azza SM, Abuelnaga EA, Elgabry A, Mansou SM. Advanced Detection of Staphylococcus aureus Enterotoxins in Milk. Global Veterinaria. 2013; 4: 403-405.

23. Moser A, Stephan R, Corti S, Johler. Comparison of genomic and antimicrobial resistance features of latex agglutination test-positive and latex agglutination test-negative Staphylococcus aureus isolates causing bovine mastitis. Journal of Dairy Science. 2013; 96: 329-334.

24. Ortega-Vinuesa, JL, Bastos-Gonzalez D. A review of factors affecting the performances of latex agglutination tests. Journal of Biomaterials Science. Polymer Edition. 2001;12: 378-408.

25. Collings AF, Caruso F. Biosensors: Recent Advances. Reports on Progress in Physics. 1997; 60: 1397-1445.

26. Cooper HM, Patterson Y. Production of polyclonal antisera. Curr Protoc Immunol. 2008; Chapter 2:Unit 2.4.1-2.4.10.